Technology in Cancer Research & Treatment
ISSN 1533-0346
Volume 3, Number 5, October (2004)

Quantitative Assessment of Tumor Vasculature and Response to Therapy in Kaposi's Sarcoma Using Functional Noninvasive Imaging

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Two noninvasive methods, thermography and laser Doppler imaging (LDI), were assessed for their ability to quantitatively assess parameters of vascularity in lesions of HIV-associated Kaposi's sarcoma (KS). Thermography and LDI images of a representative KS lesion were recorded in 16 patients and compared to normal skin either adjacent to the lesion or on the contralateral side. Eleven of the 16 patients had greater than 0.5 °C increased temperature and 12 of the 16 patients had increased flux (measured by LDI) as compared to normal skin. There was a strong correlation between these two parameters ($R = 0.81$, $p < 0.001$). In ten patients, measurements were obtained prior to therapy and after receiving a regimen of liposomal doxorubicin and interleukin-12. After 18 weeks of therapy, temperature and blood flow of the lesions were significantly reduced from the baseline ($p = 0.004$ and 0.002 respectively). These techniques hold promise to assess physiologic parameters in KS lesions and their changes with therapy.

Key words: Kaposi's sarcoma, Angiogenesis, Anti-KS therapy, Thermal imaging, Laser doppler imaging.

Introduction

It is now well recognized that new vessel formation is an essential component in tumorigenesis, and there is currently a substantial interest in developing therapies to treat cancer through inhibition of this process (1, 2). To monitor such therapy, it is desirable to establish techniques to assess tumor vasculature and changes with therapy (3). Several imaging techniques such as dynamic contrast-enhanced magnetic resonance (MR) imaging (4, 5), positron emission tomography (PET) (6, 7), computed tomography (CT) (8, 9), color Doppler ultrasound (US) (10, 11), and fluorescence imaging (8, 9) have been used in angiogenesis related research. However, all of these techniques which measure such phenomena are either invasive, require the application of the probe directly onto the tissue being studied, or expensive. There are currently no standard noninvasive techniques to assess parameters of angiogenesis in lesions of interest and to monitor changes in these parameters with therapy.

Kaposi's sarcoma (KS) represents a useful model to study anti-angiogenesis approaches and the imaging of such therapies (12, 13). KS is a frequent cause of morbidity and mortality among individuals infected with human immunodeficiency virus (HIV) (14). Pathologically, KS lesions typically have abnormal vascular slits that are lines by spindle-shaped cells (15). KS is caused in part by a gammaherpesvirus called Kaposi's sarcoma associated herpesvirus (KSHV) or human herpesvirus-8 (HHV-8) (16). This virus encodes for sever-

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al factors with angiogenic activity and can also induce production of angiogenic factors by infected cells (17-21). Cutaneous KS lesions are easily accessible for noninvasive techniques that involve imaging of tumor vasculature, and they may thus represent a model tumor in which to assess certain parameters of angiogenesis.

In an attempt to assess noninvasive methods for studying KS lesions, we have applied two noninvasive techniques: infrared thermal imaging (thermography) and laser doppler imaging (LDI). Thermography graphically depicts temperature gradients over a given body surface area at a given time. It has been used to study biological thermoregulatory abnormalities that directly or indirectly influence skin temperature (22-26). Thermography provides an integrated thermal signature which combines deep and surface sources and in general can be related to increased blood flow associated with increased metabolic activity (27-30). Thus, this approach is best used in conjunction with other imaging techniques. LDI can more directly measure the blood perfusion of small blood vessels in tissue, which generally increases as the blood supply increases during angiogenesis (31, 32). By combining thermography with LDI, it may be possible to differentiate the near surface sources from the deeper infrared sources, thus providing a useful means to assess local changes in tissue vascularization.

In this manuscript, we describe techniques for the assessment of thermography and LDI in patients with KS. In addition, we describe the use of these techniques to monitor patients undergoing an experimental anti-KS therapy.

Methods

Patients

The patients in this study all had biopsy-proven acquired immunodeficiency syndrome (AIDS)-associated KS with easily accessible cutaneous lesions. One group studied consisted of 16 male patients, 21 to 45 years of age, who had not received a specific KS therapy during the previous three weeks. Ten patients had imaging studies done at entry and week 18 as part of a treatment protocol for the study of liposomal doxorubicin and interleukin-12 (33). Protocols for the study of these patients were approved by the Institutional Review Board of the National Cancer Institute (NCI), and written informed consent was obtained from all patients. Relevant clinical information including the therapeutic regimens, overall response to therapy, and lesion measurements were extracted from the clinical and treatment protocol records.

Thermography and LDI

Most KS patients had multiple lesions on their bodies. For the study, one lesion of each patient that was at least 0.2 cm in diameter was selected. Prior to the study, the site of each lesion was measured in two perpendicular axes, and the size calculated as the product of these two axes. Lesion nodularity was assessed by touch as previously published (13).

Immediately prior to imaging, each patient removed sufficient garments and accessories to expose the lesion area, and an approximately 5 cm × 5 cm square mask was placed around the lesion to assist in providing orientation as to the area studied. Prior to, and during imaging, the subjects were seated at rest, in a closed room with temperature of approximately 23 °C and humidity of approximately 50%. Thermal patterns were recorded using an infrared camera (Thermovision Alert, Flir Systems, USA) with a uniform sensitivity in the wavelength range of 8 to 12 microns and temperature resolution less than 0.05 °C. Each thermogram comprised 320 by 240 pixels and output of each pixel consisted of a 12 bit digital signal. The instrument utilized was sensitive enough to measure temperature changes resulting from variations in blood flow. Thermal images of the lesion area, the area surrounding the lesion, and the contralateral site were assessed. The mean values and standard deviations of the temperatures were calculated by averaging pixel values over a region of interest (ROI), corresponding to the visible lesion, and comparing that to the surrounding area 3 to 4 cm from the edge of the lesion or to the contralateral site. The position of the lesion was located by matching the mask in the images with color digital photographs of the lesion area obtained prior to the measurement.

Blood perfusion was subsequently assessed in the lesion and adjacent area of the lesion including the contralateral site using LDI. The output of LDI commonly used to describe blood flow measurement is 'flux': a quantity proportional to the product of the average speed of the blood cells (often referred to as blood velocity) and their number concentration (referred to as blood volume). LDI produces a two dimensional image of tissue blood perfusion by scanning a low power solid-state laser beam over the tissue of interest. Low power light from a monochromatic stable laser, such as a 5-mW Helium-Neon laser, incident on tissue, is scattered by moving red blood cells. LDI measures the Doppler shift due to the movement of red blood cells. LDI enables noninvasive analysis of blood flow patterns in skin up to an approximate depth of one millimeter. We used a dual wavelength (MoorLDI, Moor Instrument, Inc, UK) imager for simultaneous scanning at two wavelengths, 690 nm (visible red) and 780 nm (near infrared) with spatial resolution of approximately 100 microns for assessing blood flow from differing microvascular beds (34). The LDI images were acquired by scanning the marked lesion area. The LDI value (flux) in the lesions was quantified in arbitrary units (AU). The mean values and standard deviations of the LDI images were calculated by averaging pixel values over a ROI at the center of the

lesion and comparing this to the surrounding area 3 to 4 cm from the edge of the lesion or to the contralateral site. The experiments were repeated on two separate occasions to assess the reproducibility of the results and averaged the values.

In preliminary studies, we compared the temperature and flux in the ROI to the uninvolved surrounding area 3 to 4 cm from the edge of the marked region and to an equivalent area on the contralateral appendage. We also compared uninvolved areas on both arms and legs of the same patient. These preliminary studies indicated that there was some variation between the different appendages with respect to the temperature in uninvolved areas and that the uninvolved area surrounding the ROI but within the marked area was most suitable for an uninvolved control region. Quantitative studies were thus performed comparing the ROI with the uninvolved surrounding area.

The reproducibility of temperature of the thermography and flux of the LDI systems were verified by imaging the left arm of three control subjects. The day-to-day variations in temperature and flux values were studied during three consecutive days and three times per day. The interval between the consecutive measurements within a day was 10 min. After 15-20 min of acclimatization to the room, each subject sat comfortably with their left forearm support on an arm rest at the level of the heart. Before the experiment, the position of the forearm used for the measurement was marked, and the measurement was repeated at the same position on every subsequent occasion. The temperature and LDI values were estimated by a ROI at a marked position. Data are expressed as mean ± SD (standard deviation).

Ten patients were studied at entry onto a treatment protocol of interleukin-12 (IL-12) and liposomal doxorubicin and after 18 weeks of therapy (33). The treatment regimen and preliminary clinical data for this experimental regimen have been previously described (13). Patients entered onto this study all had advanced KS, and during the first 18 weeks of therapy, they were administered liposomal doxorubicin, 20 mg/m^2 by intravenous bolus every three weeks plus interleukin-12, 300 ng/kg by subcutaneous injection twice weekly. After this induction phase, the patients were maintained on a regimen of interleukin-12, 500 ng/kg by subcutaneous injection twice weekly. The overall clinical response of KS to this treatment regimen was assessed using a minor modification of the AIDS Clinical Treatment Group (ACTG) KS parameters as previously published (13, 35). In short, responses were based on changes in the total number of KS lesions, the number of nodular KS lesions, the sum of the products of the larger perpendicular diameters of five representative "marker" lesions selected at entry onto the protocol, tumor associated edema or effusions, and visceral disease. A complete response was defined as the absence of all detectable disease for at least four weeks with a biopsy of at least one previous representative lesion showing the absence of malignant cells. Criteria for a partial response included a 50% or greater decrease in the number of lesions, complete flattening of at least 50% of nodular lesions, a 50% decrease in the sum of the products of the diameters of the marker lesions, or a 50% decrease in radiologically measurable visceral disease lasting at least four weeks. Criteria for progression were in short a 25% increase in the parameters noted above. A patient not meeting any of the criteria above was considered as having stable disease. Assessment of the clinical response of individual imaged lesions was by the ACTG criteria as they apply to individual lesions (35). In particular, a partial response was defined as a 50% or greater reduction in lesion area or a complete flattening of a previously raised lesion.

Statistical Analysis

Data are expressed as median and interquartile ranges. The correlation between temperature and LDI value (flux) was assessed using a Spearman rank correlation coefficient (nonparametric). The significance of the changes in temperature and flux during therapy was determined using the Wilcoxon signed rank test.

Results

Baseline Studies

We first assessed a group of male patients, 21 to 45 years of age, with HIV-associated KS who had not received any specific anti-KS therapy during the previous three weeks (Table I). One lesion was examined on each of the patients. All of the patients were receiving highly active anti-retroviral therapy (HAART) at the time of study. All but one of the lesions

Table I
Patient information.

Patient no.	Age (yrs.)	Site of lesion studied	Lesion size (mm^2)	Nodularity
1	38	Right leg	350	Yes
2	44	Right chest	266	No
3	36	Right leg	357	Yes
4	38	Right knee	42	Yes
5	25	Left arm near wrist	117	Yes
6	37	Right leg	100	Yes
7	34	Right chest	48	Yes
8	38	Left shoulder	130	Yes
9	34	Right arm	130	Yes
10	45	Right shin	40	Yes
11	45	Left arm	50	Yes
12	21	Right forearm	63	Yes
13	39	Left shoulder	336	Yes
14	34	Left leg	6	Yes
15	35	Left arm near wrist	165	Yes
16	43	Left calf	336	Yes

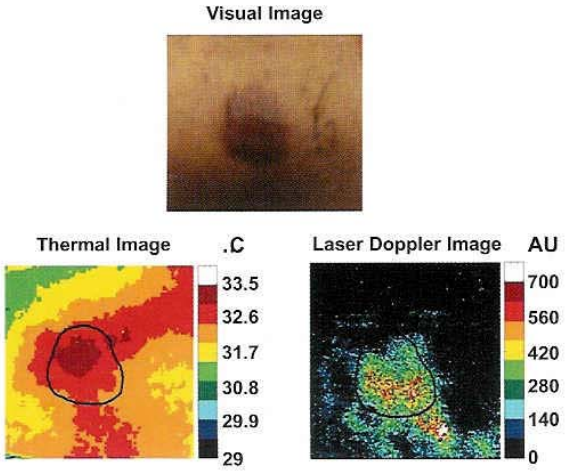

Figure 1: Typical multi-modality images obtained from a patient with KS lesion. The number '5' in the visual image was written on the skin to identify the lesion for tumor measurement. The size of the lesion was 21 × 16 mm. The solid line in the thermal and LDI demarks the border of the visible KS lesion.

were nodular and they had a median area by visual measurement of 124 mm^2 (range 6 to 336 mm^2).

An example of the images obtained from a typical KS nodular lesion using different modalities is shown in Figure 1. As can be seen in the thermal image, the temperature of the lesion was approximately 2 °C higher than that of the normal tissue adjacent to the lesion. As was the case in a number of lesions studied, the area of increased temperature extended beyond the area of the visible lesion. In some cases, the area of increased temperature followed the course of a superficial vein and was attributable to the blood in the vein. In other cases, such as the lesion depicted here, this was not associated with a superficial vein, suggesting that it was related to the tumor process. As can be seen in the LDI image of the same lesion, there was increased blood flow in the area of the lesion as compared to the surrounding tissue, with a maximum increase of over 900 AU. Unlike the thermal image, the increased blood velocity extends only slightly beyond the area of this visible lesion.

Eleven of the 16 untreated lesions had a greater than 0.5 °C elevated temperature as compared to the control area (Fig. 2). The median temperature elevation of the ROI as compared to the surrounding area was 1.1 °C (range -0.68 to 3.43 °C). In addition, 12 of the 16 lesions had increased blood velocity as assessed by LDI (median 66 AU, range -44 to 451 AU). As can be seen in Figure 2, there was a strong correlation between the temperature elevation and the LDI value in these 16 lesions (R = 0.81, p = 0.0001). Similar results were also obtained when comparing temperature and LDI values of the lesion to uninvolved contralateral sites.

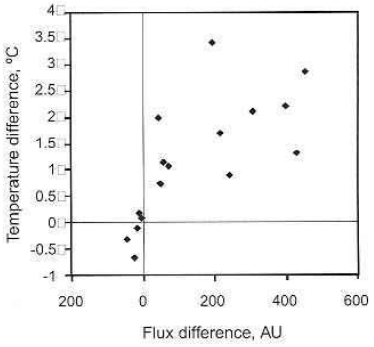

Figure 2: Relationship between the difference in temperature and flux assessed by LDI of the lesion and surrounding area of the lesion of each subject. A positive correlation was observed between these two methods (R = 0.8, $p < 0.001$).

However, in a few cases, the contralateral appendage was also affected with a lesion. We excluded such cases from our analysis. However, there was no significant correlation in this sample set between lesion size and either temperature (R = 0.46) or LDI value (R= 0.39).

To assess the reproducibility of the methodology, a subject was measured at the same site in the same environment on nine different occasions. Over the nine measurements, the temperature and LDI values ranged between 31.02 to 32.35 °C (31.57±0.5 °C, coefficient of variation 2%) and 132 to 154 AU (143±10 AU, coefficient of variation 7%) respectively. These data provide evidence that these techniques are capable of providing reproducible results. The same reproducibility of the methodology was observed on two additional subjects.

Ten patients were studied at entry and week 18 of treatment with an experimental regimen combining a cytotoxic chemotherapeutic agent (liposomal doxorubicin) and an immunologic agent with anti-angiogenic activity (interleukin-12). One of the five representative "marker" lesions

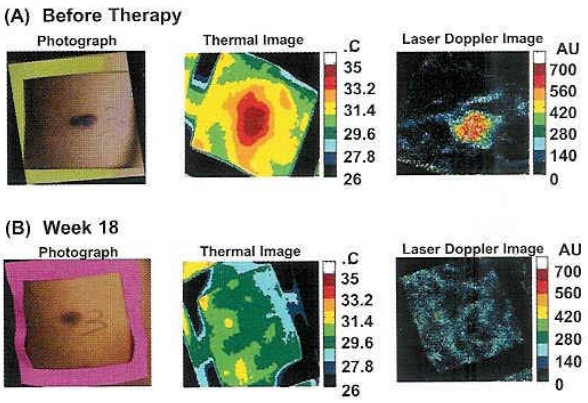

Figure 3: Typical example of lesion obtained from a subject with KS (**A**) before, and (**B**) after the treatment. The lesion becomes normal as assessed by the thermal or LDI images after 18 weeks.

Table II
Response of therapy.

Patient no.	Before Therapy		Week 18		
	Area of the Lesion mm^2	Lesion type	% reduction in area	Lesion type	Clinical Response of lesion
1	42	Nodular	71%	Flat	PR
2	117	Nodular	70%	Flat	PR
3	100	Nodular	96%	Flat	PR
4	48	Nodular	69%	Flat	PR
5	130	Nodular	3%	Nodular	SD
6	40	Nodular	-25%	Nodular	SD
7	50	Nodular	10%	Nodular	SD
8	63	Nodular	24%	Flat	PR
9	336	Nodular	-6%	Nodular	SD
10	6	Nodular	0%	Flat	PR

PR = Partial Response; SD = Stable Disease

was assessed at entry onto the protocol and the same lesion was assessed 18 weeks later. Clinical parameters for the lesions are given in Table II. Assessments of a typical lesion at entry and at week 18 are given in Figure 3. As can be seen, the lesion has increased temperature and blood velocity prior to therapy. However, although the lesion looks only minimally different by visual inspection at week 18, the temperature and flux are no longer increased as compared to the surrounding skin.

In nine of the ten patients on this regimen, the lesion studied had a decrease in temperature at week 18 of therapy (median decrease 0.99 °C, $p = 0.0039$) as shown in Figure 4A. In addition, the flux of the lesion assessed by LDI decreased in each of the 10 patients (median decrease 115 AU, $p = 0.002$) (Fig. 4B). There was a trend toward a decrease in the area of the lesions (median area was 57 mm^2 at entry and 40 mm^2 at week 18), but the change was not statistically significant ($p = 0.098$). As noted, six of the lesions met the clinical criteria for a partial response when re-imaged at week 18, while four had stable disease. It is worth noting that the temperature and LDI value decreased at week 18 both in the lesions that met the clinical criteria for a partial response at that time as well as those that did not. With regard to the overall KS response to therapy, all patients except for patient 7 met the criteria for a partial response at week 18; patient 7 subsequently met criteria for a partial response at week 30 while receiving maintenance therapy with interleukin-12 alone.

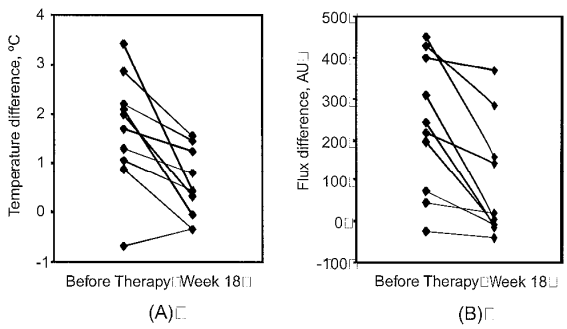

Figure 4: Temperature and LDI response of lesions of ten patients before and after treatment with liposomal doxorubicin and interleukin-12. (**A**) Temperatures of the lesions decrease significantly after the treatment ($p = 0.003$), (**B**) Blood flow of the lesions decreases significantly after the treatment ($p = 0.002$).

Discussion

There is currently an intense effort to develop novel targeted therapies such as anti-angiogenic therapy, anti-vascular therapy, immunotherapy and gene therapy for use in a variety of cancers. There is also increasing interest in approaches to monitor tumor responses that correlate or even predict the outcome of these approaches. With regard to tumor vasculature, it is desirable to develop and assess noninvasive and quantitative techniques that can not only monitor structural changes, but can also assess the functional characteristics or the metabolic status of the tumors. If successful, newly developed imaging modalities could potentially be used as predictive tools for the outcome, and therefore also used for individualization of therapeutic strategies. For anti-angiogenic therapies, factors associated with blood flow are of particular interest.

In this context, KS represents an attractive tumor in which to assess novel imaging techniques and assess its response to therapy (15, 18, 36). The lesions are cutaneous, and are thus amenable to techniques that assess superficial vasculature. Also, the lesions can be biopsied without undue risk to the patient, thus offering the potential for correlation with pathological assessment of microvessels (3).

In the current manuscript, we assess two such potential imaging techniques, infrared thermography and LDI. Both techniques are inexpensive, easy to use, and quick. Moreover, they are completely noninvasive and non-contact. We show that both of these techniques can be used to visualize KS lesions. Also, we show that although each measures an independent parameter (temperature and blood flow), they are

correlated with each other, and they revert towards normal when the KS lesions are treated with an effective regimen combining cytotoxic and anti-angiogenesis therapies.

Infrared thermal imaging is used to assess the thermal signature in the skin surface (37). Measuring skin temperature is an indirect method of assessing thermal effects due to blood flow. However, the thermal signature of skin not only reflects superficial vascularity, but also deep tissue metabolic activity (27). In this study, the temperature in the lesions was a median of 1.1 °C higher than the surrounding skin. Interestingly, in a number of lesions, the area of increased temperature extended beyond the lesion edges as assessed by visual inspection or palpation. The basis for this larger area of increased temperature is unclear at this time. It may reflect relatively deep involvement of the tumor in areas underlying normal skin. Alternatively, it may reflect inflammation surrounding the KS lesion.

LDI measures the Doppler shift due to the movement of red blood cells. Using the methodology in this study, LDI enables noninvasive analysis of blood flow patterns in skin up to an approximate depth of 1 mm (34). It can register flow in a variety of vessels including arterioles, capillaries, and venules. We found that the average value of LDI (flux) in the KS lesions was significantly higher than the surrounding tissue. These results extend a previous report of Leu *et al.* in which it was found that KS lesions had increased flux compared to normal adjacent skin (38). In the present study, the size and shape of each lesion was demarcated clearly in the LDI image, and this generally corresponded to the lesion outline as assessed by visual inspection. Moreover, LDI provides useful functional information about the supply of blood in the lesion at the tissue level. In a majority of the lesions, the flux was highest in the center of the lesion (Fig. 1). However, in some, the area of greatest flux was in the edges. The parameters accounting for these different patterns were not readily apparent, and require further study.

Comparative image analysis between LDI and thermography in the present study showed a strong correlation between these two parameters for the ROI ($p = 0.81$). However, there were some differences in individual lesions. LDI measures blood flow distribution in the superficial layer of skin of the lesion area, whereas the thermal signature provides a combined response of superficial vascularity and metabolic activity inside the tissue. These occasional discrepancies are thus not surprising. Similar results were also obtained by considering the contralateral site of the lesion as a control. However, it was hard to pick the same area of the contralateral extremity on different days for the analysis. It could have affected the results.

The vasculature of most tumors is relatively disorganized and does not fit the conventional hierarchy of arterioles, capillaries, and venules (3). KS lesions can be viewed as being particularly disorganized; the signature tumor cell is a spindle cell that forms vascular slits filled with blood cells (15). For this reason, it seemed possible at the beginning of the study that the lesions might not manifest increased blood flow as assessed by thermography or LDI. The results here, however, suggest that in spite of the tendency of KS lesions to form disorganized vascular slits, there is increased overall blood flow as compared to normal tissue. A number of techniques are now being developed to image the microvasculature of tumor biopsies (3), and it will be of interest to apply such approaches to KS and correlate the results with more functional tests as utilized here.

In the patients treated with liposomal doxorubicin plus interleukin-12, there was a substantial decrease in temperature and flux during the initial 18-week treatment period, and in some lesions, these parameters essentially normalized. In some lesions, the extent of the changes in these two parameters were greater than those assessed by measurement of tumor area, and in fact, there was no statistically significant decrease in tumor area in the lesions overall. Assessing overall responses to KS therapy is now generally performed by visually measuring and palpating the numerous lesions and using rather complex response criteria (35). However, the current tools are rather cumbersome. Also, the techniques are subject to observer variation, and this can complicate the assessment of new therapies (12). The data from the present study suggests that the techniques described here may improve with anti-KS therapy that is effective as assessed by the conventional clinical parameters of lesion size and nodularity. As such, they may be worth exploring further to assess whether they have clinical utility in the assessment of KS and in particular as to whether they may be more quantitative, sensitive, or reproducible than established techniques or even provide early information that could predict subsequent responses as assessed by more conventional parameters.

In summary, the present study demonstrates that thermography and LDI can be used to detect functional vascular abnormalities in KS lesions. The techniques are objective, easy to perform, and can detect improvement in the lesions upon administration of anti-KS therapy. The approaches may thus have utility in monitoring trials of anti-angiogenesis therapy in KS patients and possibly in predicting tumor responses. Perhaps more importantly, thermography and LDI can teach us about angiogenesis parameters of this disease, especially when utilized along with other novel approaches to assess tumor vasculature.

Acknowledgement

We thank the patients who volunteered for this study. In addition, we thank Dr. Seth M. Steinberg of the National Cancer Institute for statistical assistance and advice; Dr.

David Hattery of the Laboratory of Integrative and Medical Biophysics, NICHD; CDR Florentino Merced-Galindez of the HIV and AIDS Malignancy Branch, NCI; the data management team of the HIV and AIDS Malignancy Branch, NCI; the medical staff of the Medical Oncology Program, NCI; and the nursing, pharmacy, social work, and medical staff of the National Institutes of Health Clinical Center.

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Date Received: July 1, 2004